ELECTRON MICROSCOPY OF LIPID—PROTEIN MONOLAYERS

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ABSTRACT

Mixtures of phospholipids with β-lactoglobulin (β-lg) were spread from acidic chloroform: methanol onto the surface of a film balance, then transferred to freshly cleaved mica at 15 mN/m for examination under an electron microscope. Dipalmitoylphosphatidylcholine and dipalmitoylphosphatidic acid did not mix with β-lg and were visible as separate phases in the electron micrographs of the transferred films. Mixed monolayers containing either unsaturated lipids or dimyristoylphosphatidylcholine mixed with β-lg gave completely homogeneous micrographs with no evidence of phase separation. The results suggest that phase separation occurs in monolayers of phospholipid-β-lg when the films are prepared under conditions where the pure lipid exhibits liquid condensed behavior at the air—water interface. Homogeneous lipid—protein films apparently result when the monolayers are prepared under conditions where the lipid exhibits expanded behavior.

INTRODUCTION

Investigations of monomolecular films containing more than one chemical compound are of considerable importance because of their relevance to many natural systems. A common procedure in forming monolayers with more than one component is to spread them from a mixed solution in an appropriate spreading solvent. One question that arises is whether an intimately mixed monolayer is formed at all when a solution containing two or more materials is used to spread a film. In cases where reaction between components is detected by the deviation of some surface property from strict additivity, it is clear that some mixing must be taking place, otherwise interaction could not occur. Where no interaction between components is detected, i.e., the additivity law [1] is obeyed, care must be taken in interpretation. The results could be due to either ideal mixing of the various components of the film or to phase separation where the components are immiscible [1]. Clearly it is vital to know the degree of mixing or phase separation in multicomponent films where interactions are being studied.

Previous work established that egg yolk phosphatidic acid (EYPA) and β-lactaglobulin (β-lg) could either interact or behave ideally in monolayer

films, depending on the pH and ion content of the subphase, and an electrostatic mechanism was proposed [2]. The degree of homogeneity of the films was not clearly demonstrated although some mixing must have occurred in the cases where lipid—protein interaction was observed. In this paper we report on the electron microscopic examination of lipid—protein films of various phospholipids mixed with β-lg. The results shed some light on conditions under which phase separation occurs and suggest factors that lead to mixing of lipid with protein in monolayers.

MATERIALS AND METHODS

Materials

The fully automated recording film balance has been described [2]. Deionized double-distilled water was used for the subphase with the pH adjusted by sulfuric acid ($10^{-4}N$, pH 4) or McIlvanes buffer for pH 6 [3]. β-Lactoglobulin A was prepared by the method of Aschaffenburg and Drewry [4]. Egg yolk phosphatidic acid and dioleoylphosphatidylcholine (DOPC) were from Avanti Polar Lipids, Inc.* (Birmingham, AL). Dipalmitoylphosphatidic acid (DPPA), dipalmitoylphosphatidylcholine (DPPC), and dimyristoylphosphatidylcholine (DMPC) were from Sigma (St. Louis, MO).

Methods

Preparation of spreading solutions, spreading technique, and film transfer have all been described except that freshly cleaved mica was used in place of quartz plates for the transfer step [2]. The transfer pressure was 15 mN/m for all work reported here. The transferred films were air dried and placed in a vacuum evaporator. Platinum from Pt—carbon pellets was laid down at angle of arctan 1/5 at a distance of 7.5 cm. Carbon was deposited vertically from a distance of 10 cm. The Pt—carbon replicas were lightly scored and floated onto a clean water surface, picked up on a 200 mesh copper grid, and examined in a Zeiss 10-B electron microscope operating at 60 kV.

RESULTS

Electron micrographs of replicas of lipid, protein, and mixed monolayers transferred from the film balance to the surface of freshly cleaved mica are shown in Figs. 1 to 3 along with a replica of freshly cleaved mica alone. The transfer pressure of 15 mN/m for the monolayers was selected to give compact films without causing film collapse or redissolving of the protein

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*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

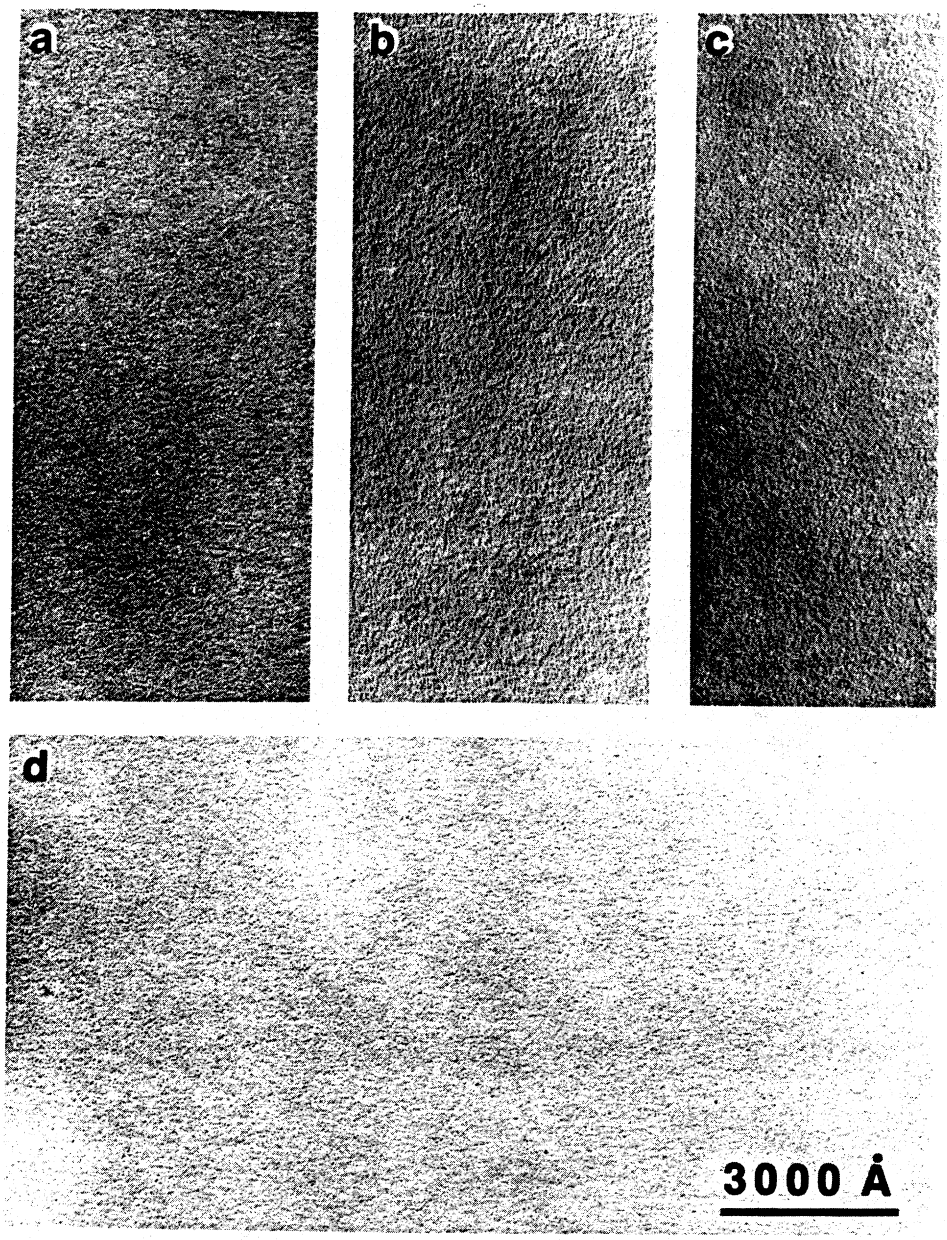

Fig. 1. Electron micrographs of (a) freshly cleaved mica with no film (b) pure EYPA monolayer, (c) pure β-lg monolayer, (d) EYPA-β-lg monolayer, 10 mole (residue) percent EYPA, pH 4 subphase.

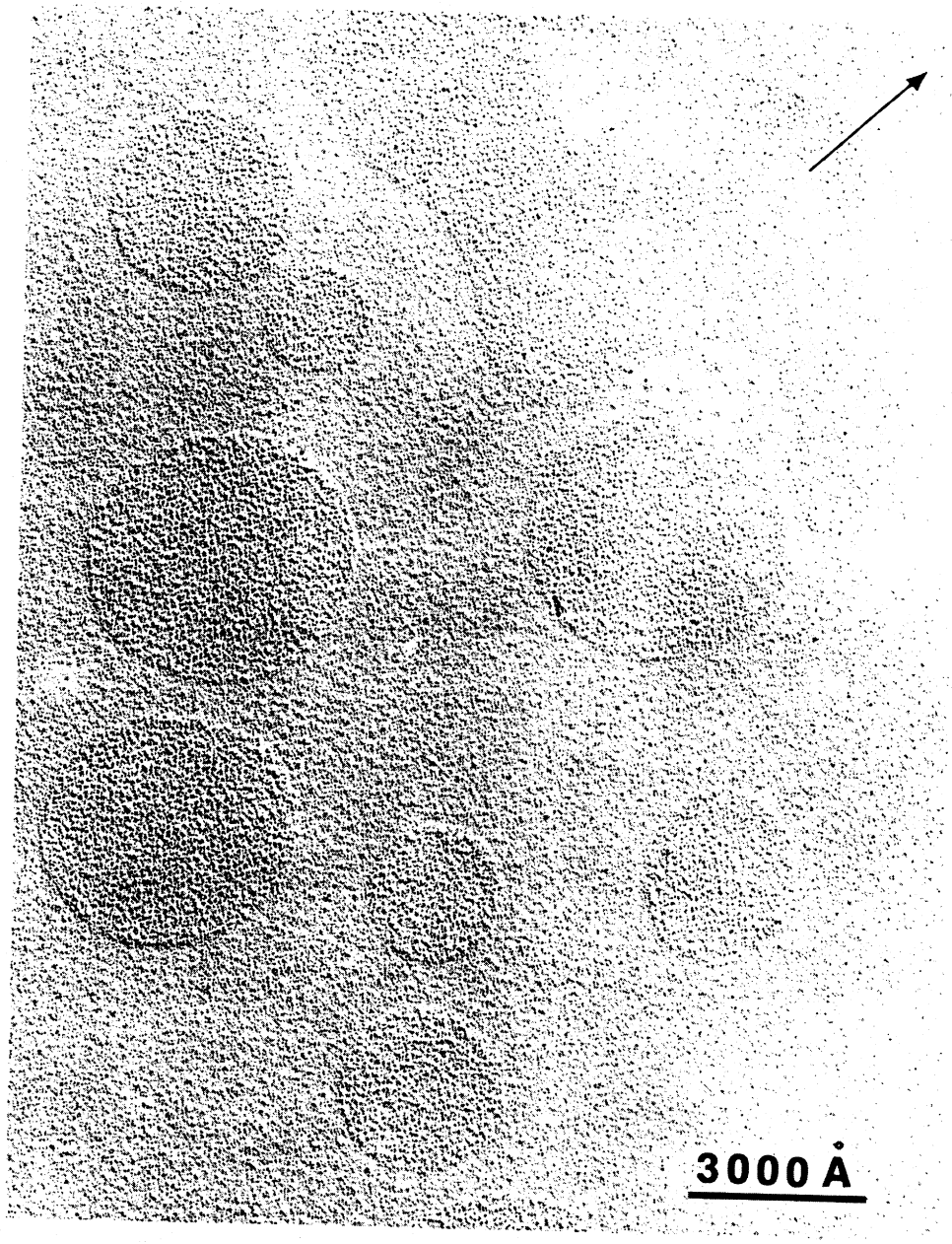

Fig. 2. Electron micrograph of a DPPA-β-lg monolayer, 18 mole (residue) percent DPPA, pH 4 subphase.

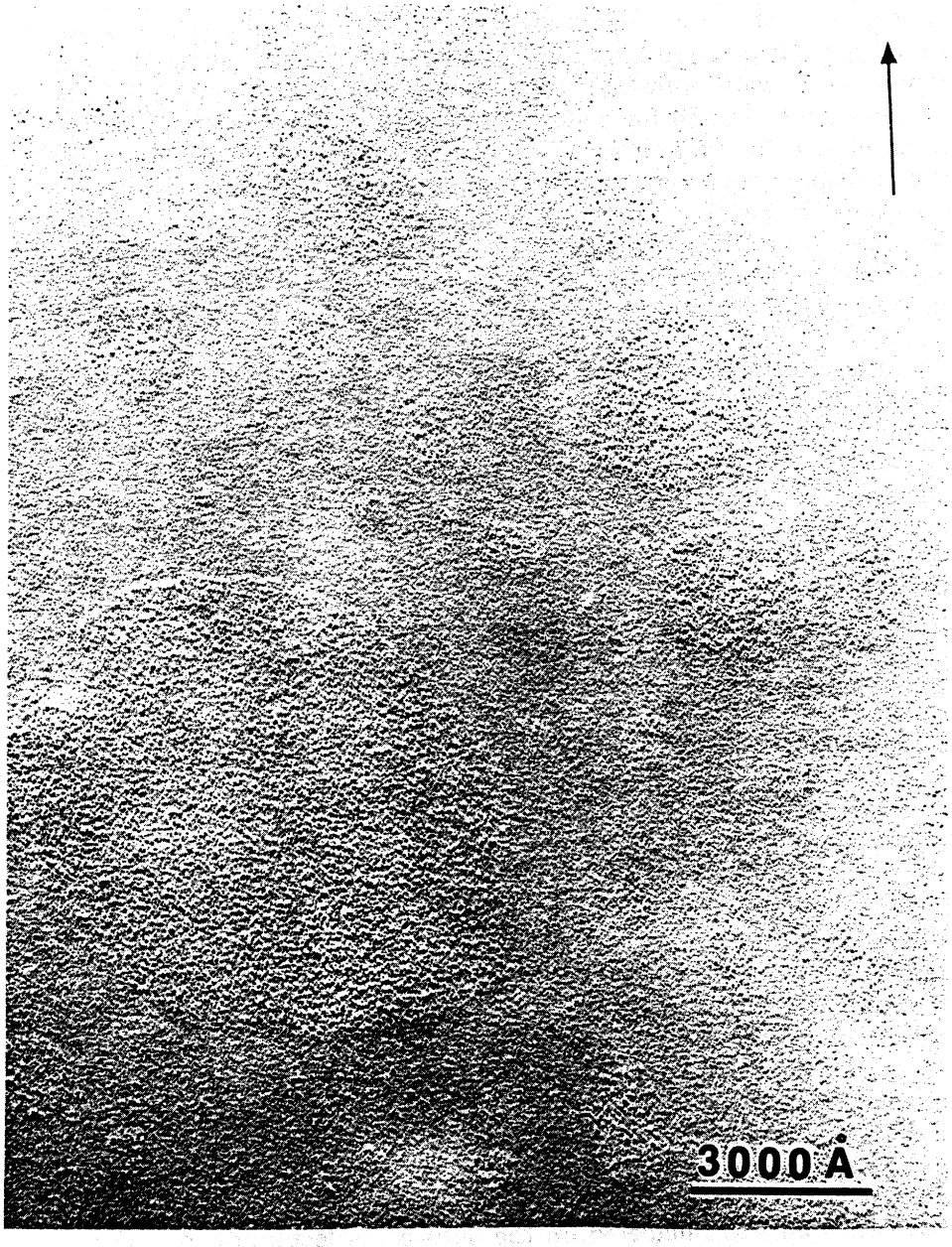

Fig. 3. Electron micrograph of a DPPC-β-lg monolayer, 20 mole (residue) percent DPPC, pH 4 subphase.

which can occur at higher pressures [5]. None of the replicas showed evidence of collapse or discontinuous gaps in the films. Transfer ratios (area reduction at air—water interface/area of mica) were from 1.0 to 1.05 for protein and lipid—protein films and 1.2 for the pure EYPA monolayer. Mica was chosen for transfer since it gives stable films free from rearrangement or collapse which was observed on some other substrates [6]. The films pictured in Fig. 1 are smooth, homogeneous, and without any discernible structure. The EYPA-β-lg film in Fig. 1 thus exhibits no evidence of separation of the mixture into distinct phases when Pt—carbon shadowing is employed. This technique is capable of resolving detail as fine as 25 Å [7].

In contrast to the micrographs in Fig. 1, the films depicted in Figs. 2 and 3 show clear evidence of phase separation or segregation of lipid from protein. Separation of lipids at different physical states was not studied in this work. The circular patches were identified as the lipid phase based on two lines of evidence, the shadowing of the replicas and the areas of the circular patches relative to the area of the entire micrographs.

The images in Figs. 1 to 3 are reverse contrast and the shadowing directions are indicated by the arrows in Figs. 2 and 3. The areas of Pt build-up are dark and the shadows (Pt deficient areas) are light. Clearly, the circular patches in Figs. 2 and 3 are raised compared to the continuous areas of the films. Assignment of the patches in Fig. 2 to DPPA and the continuous portion of the film to β-lg follows from what is known about the surface properties and size of the lipid and protein molecules. DPPA is un-ionized and exists as a condensed film on pH 4 subphases [8]. Built-up films of this type have shown the expected monolayer thickness based on a model of close-packed molecules with fully extended hydrocarbon tails standing normal to the plane of the interface [9]. A spacing of 27 Å per monolayer was found for dimyristoylphosphatidylcholine by X-ray techniques [10], which suggests a film thickness of the same order for DPPA. For β-lg, the area/residue of about 17 $Å^2$ at 15 mN/m (Fig. 1 of Ref. [2]) corresponds to 0.90 m^2/mg. Assuming a density of 1.26 g/cm^3 [11] for β-lg, a straightforward calculation yields an estimated thickness of 9 Å for a dry film. Clusters of the thicker lipid should show up as raised structures in electron micrographs of lipid—protein films where phase separation is evident.

The areas of the patches in Figs. 2 and 3 relative to the area of the film are in good agreement with calculations based on the size of the molecules at the air—water interface and the composition of the films. The composition of each film was calculated on the basis of molecules of lipid per amino acid unit (residue) of β-lg. DPPA occupies about 42 $Å^2$ per molecule [8] and β-lg about 17 $Å^2$ per amino acid residue [2] at the air—water interface at 15 mN/m. For a film containing 18 mole (residue) percent DPPA, the lipid should occupy about 35% of the total film area. The patches in three micrographs of DPPA-β-lg monolayers examined in this work occupied 32—36% of the total film area as measured by a planimeter. For DPPC in

Fig. 3 the values were 44% calculated and 40% and 51% measured on two micrographs.

The film compositions, conditions of preparation, and observations about interactions and phase separation are summarized in Table **1**. Column 1 lists the film lipid mixed with β-lg. In column 3, the film composition is listed as mole (residue) fraction, N_1, of lipid in the mixed monolayer. The results of lipid—protein interactions summarized in column 6 have been reported [2]. Mixtures of the cholines listed in Table 1 with β-lg have not been studied, but lipid—protein interactions are not expected, judged from previous work with egg yolk phosphatidylcholine [2].

TABLE 1

Summary of film balance and electron microscope observations and preparation conditions for several phospholipid-β-lactoglobulin monolayers

Lipid	Lipid[a] film type	N_1	Subphase pH	Temp.[b]	Interaction detected?	Phase separation detected?
EYPA	E	0.10	4	R.T.	yes[c]	no
EYPA	E	0.60	4	R.T.	yes[c]	no
EYPA	E	0.10	6	R.T.	no[c]	no
DPPA	C	0.18	4	R.T.	no[d]	yes
DOPC	E	0.10	4	R.T.	—	no
DPPC	C	0.20	4	21°C	—	yes
DMPC	E	0.20	4	25°C	—	no

[a]E = expanded, C = condensed.
[b]RT = 21—23°C.
[c]Reference [2].
[d]D.G. Cornell, unpublished observation.

DISCUSSION

It is interesting to note that lipids which give expanded films at the air—water interface exhibited no evidence of phase separation when mixed with β-lg in monolayers whereas the two lipids which give condensed films when pure would not mix with the protein in monolayers. This may mean that factors such as Van der Waals forces which are responsible for the close packing in condensed films of pure lipid, also contribute to the separation of the components into phases when lipids and proteins are spread together in monolayers.

The question of phase separation versus ideal mixing is an important one in monolayer studies where interactions between two or more components are being considered. In mixed films of phosphatidic acid and protein, the head groups of the lipid could be in intimate contact in a monolayer where phase separation occurs or they could be widely spaced in an homogeneous

film. It is well known that the apparent pK of an acid can be shifted by several units when the head groups are in close contact [12]. For example, the phosphoric acid groups of glycerol 2-phosphoric acid, EYPA and DPPA are identical and the pK's of the acids should be the same except for small inductive effects from the organic portion of the molecules. In practice however, pK_1 was found to be: 1.3 for glycerol 2-phosphoric acid in dilute solution [13], 3.5 for EYPA in an expanded film [8], and for DPPA, pK_1 was about 8 in a condensed film [8]. A similar result was obtained for the carboxylate ion where an apparent pK of 8.6 to 9.9 was found for films of palmitic acid compared to a pK of 4.7 in dilute aqueous solution [14].

All of the above suggests that the packing of acidic head groups in a film will have a marked influence on their acid—base behavior. For example, mixed films of EYPA and β-lg on pH 4 subphase exhibited smaller areas than would be expected from the pressure—area curves of the pure components [2]. This was explained by an electrostatic attraction between the negatively charged EYPA and the positively charged protein [2]. On the other hand, neither EYPA-β-lg films on pH 6 subphase [2] nor DPPA-β-lg films on pH 4 subphase (Table 1) exhibited lipid—protein interaction as determined by pressure—area curves. With no additional information, one could only speculate about phase separation or ideal mixing as explanations for the apparent absence of interaction. The results in Fig. 2 clearly show that lipid—protein interaction was not detected in DPPA-β-lg films because the components formed separate phases, whereas the absence of any observable structure in micrographs of EYPA-β-lg films (Table 1) suggest that these components form ideal mixtures on pH 6 subphase. This last observation lends additional support to the electrostatic mechanism discussed in ref. [2].

A similar situation exists with mixtures of β-lg with the phosphatidyl cholines. Micrographs of DPPC-β-lg films clearly show phase separation as seen in Fig. 3. Micrographs of DOPC-β-lg films however, were completely homogeneous, suggesting intimate mixing of these components as summarized in Table 1. Previous work showed that egg yolk phosphatidylcholine (EYPC) exhibited no interaction with β-lg in monolayer films [2]. Since both EYPC and DOPC are unsaturated, their surface behavior should be similar. The results obtained in this work with DOPC-β-lg suggests that EYPC-β-lg also mix ideally in monolayers.

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Since the completion of this manuscript, a lattice model of a DPPC bilayer containing integral proteins has been described [15]. The model predicts that above the gel—liquid crystal transition temperature (T_c) there is a single homogeneous phase while for temperature below T_c the system separates into an essentially pure lipid phase and a protein-rich phase containing lipid. The results shown in Figs. 1 to 3, summarized in Table 1 above, are consistent with these predictions since above T_c lipids exhibit expanded behavior at the air—water interface whereas for temperatures sufficiently far below T_c, condensed behavior is observed.

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